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The thermolabile variant of 5,10-methylenetetrahydrofolate reductase is a possible risk factor for amyotrophic lateral sclerosis

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Abstract: Abstract Hyperhomocysteinemia is a risk factor for neurodegeneration, and binding of copper by homocysteine is a putative underlying mechanism. As mutations of the copper-dependent superoxide dismutase are observed in familial ALS, we tested whether genetic variants with influence on homocysteine metabolism are associated with ALS. We compared the frequency of seven variants of genes involved in homocysteine metabolism in 162 patients with sporadic ALS and 162 controls who did not significantly differ in age ($t = 1.27$, $p = 0.205$) and gender ($\chi^2 = 2.48$, $p = 0.115$) using binary regression analysis. Results showed that the variant MTHFR c.677C>T was significantly associated with ALS, i.e. the T-allele was more frequent among patients. Explorative regression analysis revealed that MTHFR c.677C>T was not associated with spinal ALS, but with bulbar onset: CC/CT/TT in patients 0.33/0.51/0.16 versus 0.50/0.44/0.06 in controls; Wald = 5.73, $p = 0.017$. In addition, DHFR c.594+59del19bp was not associated with spinal, but with bulbar onset: del,del/del,ins/ins,ins in patients 0.16/0.67/0.18 versus 0.11/0.52/0.37 in controls; Wald = 5.02, $p = 0.025$. The other variants did not show significant associations. In summary, the variants MTHFR c.677C>T and DHFR c.594+59del19bp are involved in homocysteine metabolism. Homocysteine is neurotoxic and binds copper. Thus, the individual variability of homocysteine metabolism, e.g. due to genetic variants, may contribute to the vulnerability of ALS.

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The thermolabile variant of 5,10-methylenetetrahydrofolate reductase is a possible risk factor for amyotrophic lateral sclerosis

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Abstract

Background: Hyperhomocysteinemia is a risk factor for neurodegeneration, and binding of copper by homocysteine is a putative underlying mechanism. As mutations of the copper-dependent superoxide dismutase are observed in familial ALS, we tested whether genetic variants with influence on homocysteine metabolism are associated with ALS.

Subjects and Methods: We compared the frequency of seven variants of genes involved in homocysteine metabolism in 162 patients with sporadic ALS and 162 controls who did not significantly differ in age ($t=1.27$; $p=0.205$) and gender ($\chi^2=2.48$; $p=0.115$) *using binary regression analysis*.

Results: The variant MTHFR c.677C>T was significantly associated with ALS, i.e., the T-allele was more frequent among patients. Explorative regression analysis revealed that MTHFR c.677C>T was not associated with spinal ALS, but with bulbar onset: CC/CT/TT in patients 0.33/0.51/0.16 versus 0.50/0.44/0.06 in controls; Wald=5.73; $p=0.017$. In addition, DHFR c.594+59del19bp was not associated with spinal, but with bulbar onset: del,del/del,ins/ins,ins in patients 0.16/0.67/0.18 versus 0.11/0.52/0.37 in controls; Wald=5.02; $p=0.025$. The other variants did not show significant associations.

Discussion: The variants MTHFR c.677C>T and DHFR c.594+59del19bp are involved in homocysteine metabolism. Homocysteine is neurotoxic and binds copper. Thus, the individual variability of homocysteine metabolism, e.g. due to genetic variants, may contribute to the vulnerability of ALS.

Key words: Amyotrophic lateral sclerosis, methylenetetrahydrofolate reductase, homocysteine, SOD.

Introduction

Amyotrophic lateral sclerosis (ALS) is characterized by progressive degeneration of upper and lower motor neurons in motor cortex, brainstem and spinal cord leading to progressive limb weakness, bulbar palsy and respiratory insufficiency. Spinal onset is observed in the majority of cases, but bulbar onset is seen in up to 30%. In later disease stages almost all patients demonstrate bulbar involvement. The mean survival time is 3-5 years. Respiratory failure is the major life-limiting factor (1-3). Most cases are sporadic (sALS), but about 10% are familial (fALS). About 15-20% of autosomal dominant fALS patients show mutations in the copper-dependent superoxide dismutase gene (*SOD1*) (4,5). Our previous finding of hyperhomocysteinemia leading to copper binding, deficiency of cytochrome oxidase C and consequently apoptosis in different cell lines including primary rat neurons prompted us to test whether variants of genes involved in homocysteine metabolism are associated with the development of ALS (6,7). We analysed the frequency of seven genetic variants of homocysteine metabolism in a case-control-study. Further, explorative analyses were performed to investigate whether these variants are associated with the clinical presentation of ALS.

Subjects and Methods

Serial Caucasian adult patients with sporadic ALS were recruited at the Departments of Neurology of the University Hospitals of Ulm, Germany, and Zurich, Switzerland. Patients were diagnosed to have definite or probable sporadic ALS by modified Airlie House El Escorial criteria (8). In brief, diagnosis was based on clinical examination, electromyography and nerve conduction studies. Cranial and cervical magnetic resonance imaging were performed when considered necessary. Electromyography (EMG) showed signs of anterior horn cell degeneration whereas nerve conduction studies were without signs of other underlying neurological disorders. All patients underwent routine biochemical tests including thyroid hormones, serum protein electrophoresis, complete blood count and standard CSF analysis. Healthy age- and gender-matched Caucasian subjects, e.g., partner of patients

without any history of neurological disease, served as controls. DNA sampling and genetic analyses were approved by the local Ethics Committees. Written informed consent for genetic analysis was obtained from each individual.

In total, 162 patients and 162 controls were enrolled (table 1). Patients and controls did not significantly differ in age ($t = 1.27$; $p = 0.205$) or gender (Chi = 2.48; $p=0.115$).

Genomic DNA was prepared from peripheral leukocytes and was used for genotyping of seven variants of genes involved in homocysteine metabolism by allele specific PCR amplification or by PCR amplification with subsequent restriction analysis (9):

- cystathionine beta-synthase (CBS) c.844_855ins68 (splice alteration affecting the transcript level; GenBank S78267.1)
- dihydrofolate reductase (DHFR) c.594+59del19bp (intronic deletion supposedly affecting the transcript level; GenBank NM_000791.3)
- MTHFR c.677C>T (missense mutation p.A222V; rs1801133)
- MTHFR c.1298A>C (missense mutation p.E429A; rs1801131)
- methionine-homocysteine S-methyltransferase (MTR; “methionine synthase”) c.2756A>G (missense mutation p.D919G; rs1805087)
- reduced folate carrier 1 (RFC1) c.80G>A (missense mutation p.R27H; rs1051266)
- transcobalamin 2 (Tc2) c.776C>G (missense mutation p.P259R; rs1801198)

Genotyping was successful for all DNA samples. A χ^2 goodness-of-fit analysis was performed to analyse whether the distribution of genotypes was in disequilibrium with the Hardy-Weinberg-equation in the controls. Due to a Hardy-Weinberg disequilibrium in the controls ($p<0.001$), the variant RFC1 c.80G>A was excluded from further analyses. Age and gender were compared between groups by an independent t-test or Pearson's χ^2 -test. To analyse independent associations between genetic variants and clinical parameters and to avoid artifacts due to multiple testing or due to any heterogeneity concerning age and gender between patients and controls, we used multivariate analyses, i.e., binary regression with all genotypes, age and gender as covariables and diagnosis “ALS” versus “control” as dependent variable to analyse associations of genotypes with the incidence of ALS as

primary parameter of interest. Exploratively, the genotypes together with age and gender as covariables were analysed with age of onset or, alternatively, disease duration as dependent variable in linear regression analysis. In addition, binominal regression analysis with all genotypes, age and gender as covariables was used to analyse the association of genotypes with subtypes of the clinical presentation defined by bulbar versus spinal disease onset. Finally, explorative analyses investigated subgroups defined by age and gender.

Results

The results of the multivariate binominal regression analysis of the genetic variants with age and gender as covariables are shown in table 2.

The variant MTHFR c.677C>T was significantly associated with ALS, i.e., the T-allele was more frequent in patients than in controls with 6% carriers of the T-allele in homozygous state in the controls compared with 13% in the patients (Wald=5.39; p=0.020). The other genetic variants did not show such associations. Explorative analyses did not reveal further significances when subgroups were analyzed defined by gender or age. None of the genetic variants was associated with age of onset or disease duration. However, explorative binary regression analysis showed that MTHFR c.677C>T was not significantly associated with ALS with spinal onset of disease (CC/CT/TT in patients: 0.43/0.45/0.12, and 0.50/0.44/0.06 in controls; Wald = 3.14; p = 0.076), but with bulbar onset (CC/CT/TT in patients: 0.33/0.51/0.16, and 0.50/0.44/0.06 in controls; Wald = 5.73; p=0.017). In addition, DHFR c.594+59del19bp was not associated with spinal onset ALS (del,del/del,ins/ins,ins in patients: 0.14/0.46/0.40, and 0.11/0.52/0.37 in controls; Wald = 0.141; p = 0.707), but with bulbar onset ALS (del,del/del,ins/ins,ins in patients: 0.16/0.67/0.18, and 0.11/0.52/0.37 in controls; Wald = 5.02; p=0.025).

Discussion

In our study sample, the MTHFR variant c.677C>T was associated with sporadic ALS and thus may be a risk factor for the development of this disease. If subgroups were analyzed, this association was only significant for bulbar onset ALS, but not for spinal ALS. The

thermolabile MTHFR variant, caused by the T-allele of MTHFR c.677C>T, reduces MTHFR activity yielding elevated homocysteine plasma levels (10). Elevated homocysteine plasma levels are a risk factor for neurodegeneration. Binding of copper by homocysteine and an impairment of copper-dependent enzymes like cytochrome C oxidase and superoxide dismutase 1 (SOD1), which play a role in familial ALS, may be underlying mechanisms (6). Thus, on a genetic basis, homocysteine metabolism, speculatively via its influence on copper homeostasis, may contribute to ALS pathogenesis. However, we cannot estimate to what extent our data depend on our study population. Due to the limitations of any association study, these results must be re-tested in independent study samples, especially since the frequency of the TT genotype of MTHFR c.677C>T in the control group was lower than expected (11-13). Also, the association of MTHFR c.677C>T with bulbar ALS only, which was observed in secondary explorative subgroup analyses, and the association of DHFR c.594+59del19bp with bulbar ALS should be re-tested. The lack of association of the genetic variants with the clinical course argues against a major influence of homocysteine metabolism on ALS outcome. However, in the absence of a curative therapy approach yet, a confirmed association of elevated homocysteine plasma levels with ALS development may lead to novel prophylactic or therapeutic strategies, as reduction of homocysteine plasma levels can be easily accomplished (7).

Disclosure: All authors report no conflicts of interest.

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Table 1. Demographic data. Disease duration means time from disease onset until death or tracheotomy. 14 patients from Ulm who were still alive at genetic analysis were excluded from analyses. Data for enrolment, gender distribution and type of onset are given as percent. Data for age and disease duration are given as mean \pm standard deviation (SD).

	patients (n=162)	controls (n=162)
enrolled in Ulm / Zurich	0.73 / 0.23	0.72 / 0.24
female / male gender	0.33 / 0.67	0.39 / 0.61
spinal / bulbar onset	0.67 / 0.33	--
age (patients: at onset) in years \pm 1 sd (range) ¹	60 \pm 12 (24-92)	59 \pm 15 (18-88)
disease duration in months \pm 1 sd (range)	50.7 \pm 39.3 (6-332)	--

Table 2: Association of genetic variants of homocysteine metabolism with ALS.

Variant	Genotype			Wald; p
CBS c.844_845ins68bp	del/del	del/ins	ins/ins	
patients	0.87	0.11	0.01	0.47; 0.495
controls	0.81	0.19	0	
DHFR c.594+59del19bp	del/del	del/ins	ins/ins	
patients	0.14	0.52	0.33	0.80; 0.372
controls	0.11	0.52	0.37	
MTHFR c.677C>T	CC	CT	TT	
patients	0.40	0.48	0.13	5.39; 0.020
controls	0.50	0.44	0.06	
MTHFR c.1298A>C	AA	AC	CC	
patients	0.49	0.41	0.10	0.19; 0.546
controls	0.48	0.41	0.11	
MTR c.2756A>G	AA	AG	GG	
patients	0.69	0.30	0.01	0.39; 0.530
controls	0.67	0.29	0.04	
TC2 c.677C>G	CC	CG	GG	
patients	0.33	0.46	0.21	0.21; 0.646
controls	0.30	0.44	0.26	